Plasma deoxysphingolipids: a novel class of biomarkers for the metabolic syndrome?

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Abstract: AIMS/HYPOTHESIS: Sphingolipid synthesis is typically initiated by the conjugation of L-serine and palmitoyl-CoA, a reaction catalysed by serine palmitoyltransferase (SPT). SPT can also metabolise other acyl-CoAs (C(12) to C(18)) and other amino acids such as L-alanine and glycine, giving rise to a spectrum of atypical sphingolipids. Here, we aimed to identify changes in plasma levels of these atypical sphingolipids to explore their potential as biomarkers in the metabolic syndrome and diabetes. METHODS: We compared the plasma profiles of ten sphingoid bases in healthy individuals with those of patients with the metabolic syndrome but not diabetes, and diabetic patients (n = 25 per group). The results were verified in a streptozotocin (STZ) rat model. Univariate and multivariate statistical analyses were used. RESULTS: Deoxysphingolipids (dSLs) were significantly elevated ($p = 5 \times 10$) in patients with the metabolic syndrome (0.11 ± 0.04 mol/l) compared with controls (0.06 ± 0.02 mol/l) but did not differ between the metabolic syndrome and diabetes groups. Levels of C(16)-sphingosine-based sphingolipids were significantly lowered in diabetic patients but not in patients with the metabolic syndrome but without diabetes ($p = 0.008$). Significantly elevated dSL levels were also found in the plasma and liver of STZ rats. A principal component analysis revealed a similar or even closer association of dSLs with diabetes and the metabolic syndrome in comparison with the established biomarkers. CONCLUSIONS/INTERPRETATION: We showed that dSLs are significantly elevated in patients with type 2 diabetes mellitus and non-diabetic metabolic syndrome compared with healthy controls. They may, therefore, be useful novel biomarkers to improve risk prediction and therapy monitoring in these patients.

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Plasma deoxysphingolipids – a novel class of biomarkers for the metabolic syndrome?

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Abstract:

Aims/hypothesis: Sphingolipid synthesis is typically initiated by the conjugation of L-serine and palmitoyl-CoA, a reaction catalyzed by the serine-palmitoyltransferase (SPT). SPT can also metabolize other acyl-CoAs (C_{12} to C_{18}) and other amino acids such as L-alanine and glycine giving rise to a spectrum of atypical sphingolipids. Here, we aimed to identify changes in plasma levels of these atypical sphingolipids to explore their potential as biomarkers in the metabolic syndrome and diabetes.

Methods: We compared the plasma profile of ten sphingoid bases in healthy individuals to patients with the metabolic syndrome but no diabetes and diabetic patients (n=25 per group). The results were verified in an STZ rat model. Univariate and multivariate statistical analysis was used.

Results: Deoxysphingolipids (dSLs) were significantly elevated (p= 5E-6) in patients with the metabolic syndrome (0.11 µmole/l ±0.04) compared to controls (0.06 µmole/l ±0.02) but did not differ between the metabolic syndrome and diabetes groups whereas C_{16}-sphingosine based sphingolipids were significantly lowered in diabetic patients but not in patients with the metabolic syndrome without diabetes (p=0.008). Significantly elevated dSL levels were also found in plasma and liver of the STZ rats. A principal component analysis revealed a similar or even closer association of deoxysphingolipids with diabetes and the metabolic syndrome in comparison to the established biomarkers.

Conclusions/interpretation: We showed that deoxysphingolipids are significantly elevated in patients with type 2 diabetes mellitus and non-diabetic metabolic syndrome compared to healthy controls. They might, therefore, be useful novel biomarkers to improve risk prediction and therapy monitoring in these patients.

Keywords: deoxysphingolipids, atypical sphingolipids, metabolic syndrome, type 2 diabetes mellitus, biomarker

Abbreviations: The nomenclature used in this paper generally conforms to the recommendations of the IUPAC-IUB Comission of Biochemical Nomenclature [1] C_{16}SO or C_{16} D-*erythro*-sphingosine, (2S,3R,4E)-2-amino-4-hexadecen-1,3-diol; C_{16}SA or C_{16}-D-*erythro*-sphinganine, (2S,3R,4E)-2-aminohexadecan-1,3-diol; C_{17}SO, (2S,3R,4E)-2-amino-4-heptadecen-1,3-diol; C_{17}SA, (2S,3R,4E)-2-aminoheptadecan-1,3-diol; C_{18}SO , (2S,3R,4E)-2-amino-4-octadecen-1,3-diol; C_{18}SA, (2S,3R,4E)-2-aminooctadecan-1,3-diol; C_{20}SO, (2S,3R,4E)-

2-amino-4-icosen-1,3-diol; C_{20}SA, (2S,3R,4E)-2-aminoicosan-1,3-diol, SPT, serine-palmitoyltransferase, dSL, deoxysphingolipids

Introduction:

The pathogenesis of diabetes mellitus type 2 is characterized by the development of insulin resistance, frequently because of excess body fat, that is initially overcome by increased insulin secretion and – after several years - a subsequent decrease in the functional pancreatic β-cell mass that can no longer compensate for insulin resistance [2-4]. Insulin resistance or the compensating hyperinsulinemia lead to the manifestation of a cluster of risk factors like hyperglycemia, hypertrigylceridemia, low plasma levels of high density lipoprotein (HDL) cholesterol and arterial hypertension which has been termed the metabolic syndrome. Although much progress [5] has been made in the understanding of the mutual relationships between obesity, insulin resistance, type 2 diabetes and atherosclerosis, the complete picture remains elusive. In the last years the carbohydrate centered view of the pathogenesis of diabetes has widened to include different classes of lipids [5-7] and inflammatory factors [8]. Mounting evidence suggests that sphingolipids play a role in the pathogenesis of insulin resistance and diabetes [9-11]. In particular, ceramides have been suggested to be important in the development of insulin resistance. For instance, inhibition of ceramide synthesis restored insulin sensitivity in palmitate-induced insulin resistance in mice [12].

Sphingolipids comprise a heterogeneous class of lipids that are derived from the aliphatic amino-alcohol sphingosine, which is commonly formed from the precursors L-serine and palmitoyl-CoA (suppl. Fig. 1). This reaction is catalyzed by the enzyme serine palmitoyltransferase (SPT). Besides these substrates, SPT metabolizes also other acyl-CoAs with a carbon chain length in the range of C_{12} to C_{18} [13]. Moreover, SPT shows also flexibility towards the use of other amino acid substrates such as L-alanine or glycine [14, 15] which generates an atypical category of 1-deoxy-sphingoid bases. Whereas the conjugation of alanine forms deoxy-sphinganine (doxSA), the use of glycine results in the formation of deoxymethyl-sphinganine (doxmethSA). These two metabolites can be N-acylated to form deoxysphingolipids (dSL) but not further metabolized to complex sphingolipids due to the missing C_1-hydroxyl group [16]. Consequently, those metabolites cannot be degraded by the

canonical sphingolipid degradation pathway which requires the formation of sphingosine-1-phosphate (S1P) as a catabolic intermediate. Low dSL levels are ubiquitously found in human plasma but levels are greatly increased in HSAN1 (OMIM162400), an inherited sensory neuropathy which is caused by missense mutations in SPT [15].

Physiologically, de-novo sphingolipid synthesis represents a metabolic cross point which interconnects lipid, amino acid and thereby indirectly also carbohydrate metabolism. Fluctuations in fatty acid and thereby acyl-CoA concentration are reflected in an altered carbon chain compositions of the sphingoid bases [12, 17]. Another node connects serine and alanine metabolism to the sphingolipid metabolism. Serine is formed from 3-phosphogylcerate whereas alanine can be converted from and to pyruvate through alanine aminotransferase (ALT) in a reversible transaminase reaction. The precursors for both amino acids are hence generated during glycolysis and thereby provide a functional link between sphingolipid and carbohydrate metabolism. In this respect, it is noteworthy that alanine is the major gluconeogenic amino acid with an important role in obesity and diabetes [18, 19].

In the light of these metabolic connections and the increasing evidence that certain sphingolipid molecules play a role in the pathogenesis of insulin resistance and diabetes, we compared the plasma sphingoid base profile of healthy humans to patients with the metabolic syndrome but no diabetes and patients with type 2 diabetes mellitus. The observed differences were further confirmed in a diabetic animal model.

2. Methods:

2.1 Patients:

A sex- and age-matched nested cohort of 25 patients with non-diabetic metabolic syndrome, 25 patients with metabolic syndrome and type 2 diabetes mellitus, and 25 controls without diabetes and metabolic syndrome was selected from a previously described study cohort [20] of the VIVIT institute (Feldkirch, Austria). The samples were derived from a larger cohort of unselected Caucasian patients undergoing coronary angiography for the evaluation of suspected coronary artery disease. Only patients with negative angiographic results were enrolled in this study. Age range was 55 - 69 years. The angiographers were not aware of plasma sphingolipid levels. The study was approved by the

Ethics Committee of the University of Innsbruck and all participants gave written informed consent.

According to NCEP-ATPIII criteria, non-diabetic metabolic syndrome was diagnosed if three or more of the following criteria were fulfilled: waist circumference >102 cm in men and >88 cm in women, triglycerides ≥ 1.7 mmol/l (150 mg/dl), HDL cholesterol < 1.0 mmol/l (40 mg/dl) in men and <1.3 mmol/l (50 mg/dl) in women, blood pressure ≥130 / ≥85 mmHg, and fasting glucose ≥6.1 mmol/l (110 mg/dl) but <7 mmol/L. Type 2 diabetes mellitus was diagnosed by either fasting glucose levels ≥ 7 mmol/l (126 mg/dl), or plasma glucose levels ≥ 11.1mmol/l (200 mg/dl) 2h after an oral challenge with 75g glucose or previously diagnosed diabetes. Controls were defined by the absence of both non-diabetic metabolic syndrome and type 2 diabetes mellitus

2.2 Clinical Chemistry

Venous blood samples were collected after an overnight fast of at least 12 hours before angiography was performed, and laboratory measurements were performed from fresh serum samples. The serum levels of triglycerides, total cholesterol, and high density lipoprotein (HDL) cholesterol were determined by using enzymatic hydrolysis and precipitation techniques (Triglycerides GPO-PAP, CHOD/PAP, QuantolipLDL, QuantolipHDL; Roche, Basel, Switzerland) on a Hitachi-Analyzer 717 or 911. Glycated hemoglobin (HbA1c) was determined by high-performance liquid chromatography on a Menarini-Arkray KDK HA 8140 (Arkray KDK, Kyoto, Japan). Clinical chemistry parameters were measured on a Hitachi 717 or 911 system (Roche, Basel, Switzerland).

2.3 Animal model

Male Sprague Dawley rats (180-200 g, Charles River, Calco, Italy) were housed in groups of two rats. Animal room temperature and relative humidity were set at 22±2°C and 55±10%. Artificial lighting provided a 12 hours light/12 hours dark cycle (7 a.m. – 7 p.m.). The animals had free access to diet and water. Diabetes was induced in rats fasted overnight by a single intraperitoneal (i.p.) injection of 60 mg/kg of STZ dissolved in sodium citrate buffer (pH 4.5). The control rats were injected with vehicle. Hyperglycemia was confirmed by measuring glycosuria 72 h after STZ injection, using Keto-Diabur test 5000 strips (Roche Diagnostics Spa, Italy). Only animals with glycosuria > 5% were classified as diabetic and included in the study.

Body weight and blood glucose concentration, determined by tail bleeding using strips (Ascensia Elite; Bayer, Basel, Switzerland), were measured weekly. Immediately after sacrificing the animals, liver and muscle (Gastrocnemius) were carefully dissected out and immediately frozen in liquid N_2.

2.4 Quantification of sphingoid bases

The lipids were analyzed as described before [15, 21]. Tissue samples were homogenized in lysis buffer (25mM Hepes pH8, 0.2% TX100) using Precellys® 24 tissue homogenizer (Bertin technologies, Montigny-le-Bretonneux, France). Briefly, 100µl plasma or tissue homogentate containing 80 µg extracted protein were added to 1ml of extraction buffer (2:1) methanol-KOH/chloroform and spiked with 200 pmoles of the internal standard C_{20} sphinganine (Avanti Polar Lipids).

The extracted dried lipids were acid hydrolyzed using methanolic HCl (1N HCl/10M water in methanol) and heat (65 ^{o}C, 16 h) to release the sphingoid base backbones which was followed by a second extraction. LC/MS analysis was performed as described earlier [15]. Lipids were separated on a C_{18} column (Uptispere 120 Å, 5µm, 125 × 2 mm, Interchim, France) and analyzed by an MS detector (LCQ, Thermo Inc.). The samples were measured as singletons for each subject. Inter- and intra-assay CV% was between 5% and 20% for each sphingoid base.

Analyzed sphingoid bases included C_{16}SO, C_{16}SA, C_{17}SO, C_{17}SA, C_{18}SO, C_{18}SA, C_{20}SO, C_{18}SA-diene, doxSA, and doxSO.

2.5 Statistics:

Mean comparison and ROC curve analyses

The original dataset was imported into SPSS 16.0 (SPSS Switzerland AG, Zurich, Switzerland). Since some of the measured variables did not follow normal distribution, even after transformations, non-parametric tests were used. Kruskal- Wallis test was used to compare all three groups. Mann-Whitney U test was used for comparisons between two groups and then followed by the Bonferroni correction. Spearman correlations were calculated. Receiver Operating Characteristic (ROC) curve analysis was performed and the two-tailed asymptotic p value was calculated for the ROC areas under the curve.

Orthogonal partial least square- discriminant analysis (OPLS-DA)

OPLS-DA [22, 23] was used as a multivariate predictive and regression method which is based on the concepts of principal component analysis (PCA). In PCA, a large number of correlated variables e.g. clinical, clinical chemistry and sphingolipid variables are summarized into a smaller number of uncorrelated ones called principal components. Thus, the correlation structures are preserved while reducing the number of variables. In contrast to PCA, which does not consider class membership, in OPLS-DA the group information is included (control, the non-diabetic metabolic syndrome and diabetes) as a Y variable. OPLS-DA, therefore, summarizes the data into a predictive component maximizing the between-group variation and an orthogonal component(s) describing the within-group variation. The predictive component describes the variations in X which correlate with Y. The orthogonal component describes the systematic variations within each group which do not correlate with Y, thereby removing the noise data from the predictive component.

For any modeling technique, model evaluation is necessary before any inferences are made. R^2Y reports the fit of the model to the original dataset and Q^2Y reports the predictive ability of the model calculated by cross validation. Cross validation (CV) is performed by dividing the whole dataset into multiple random groups and then predicting the class assignment of members of each group at a time.

The dataset was imported into SIMCA-P+ ®12.0.1.0 (Umetrics Inc., Umeå, Sweden) for the calculations. The groups (control, non-diabetic metabolic syndrome and diabetes) were assigned as classes. Variables which did not follow the normal distribution were log transformed before model fitting. Since some variables did not follow the normal distribution after log transformation, models were fitted twice, once with the log-transformed variables and another time without any transformations. The model evaluation parameters and interpretations did not differ in both cases. Therefore, the values for all the variables without any transformations were used. As the values for the variables had different scales, the dataset was normalized in unit variance (UV) and centered around the mean. OPLS-DA models were fitted for the classes to get the highest R^2Y and Q^2Y values. Models were done to compare 2 groups only at a time (control vs. non-diabetic metabolic syndrome and non-diabetic metabolic syndrome vs. diabetes). We refrained from the use of the three groups as a Y variable since it made the interpretations of the model results more complex.

Since PCA-based methods are sensitive to outliers, Hotelling`s $T^2$ and distance to the model, DmodX, were utilized to detect outliers. Hotelling`s T^2 is a generalization of the t distribution for the multivariate case and is usually visualized in score plots. Score plots show on the x axis the scores of the predictive component describing the between-group variation and on the y axis the scores of the orthogonal component describing the within-group variation. Tolerance ellipse is drawn in score plots outlining the 95% probability of the Hotelling's T^2 distribution. Scores for observations situated outside the tolerance ellipse can be considered as outliers. Moreover, DModX shows the distance to the model in the X space in a way similar to the residuals in the linear regression models. A critical value D-Crit of 0.05 was set as a limit for outlier detection. After outliers were detected, models were fitted twice, excluding and including the outliers. In the current study, no difference was found in the model evaluation parameters or any of the results upon inclusion or exclusion of the outliers. Therefore, models without outlier removal were used for the interpretation.

Cross validation (CV) was performed for each model with 7 groups. The CV-ANOVA was calculated along with misclassification tables and Fisher`s probabilities

Results:

For this study, we analyzed plasma samples of patients from three pre-defined subgroups each consisting of 25 sex- and age-matched patients with either manifest diabetes mellitus type 2, metabolic syndrome but no manifest diabetes mellitus, or without diabetes mellitus or metabolic syndrome (controls). Results are summarized in Table 1.

Sphingoid bases in plasma are usually N-aceylated but also conjugated with different headgroups giving rise to a great variety of different sphingolipid species. To analyze the sphingoid base composition of these metabolites we subjected the extracted lipids a sequential acid and base hydrolysis to remove the N-acyl chain and head group. The resulting free sphingoid bases were analyzed by LC-MS.

The most abundant sphingoid base in human plasma was C_{18}-Sphingosine (C_{18}SO) followed by C_{18}SA-diene, C_{16}-Sphingosine (C_{16}SO) and C_{17}-Sphingosine (C_{17}SO). Deoxy-sphingolipids were generally recovered as quantitatively minor fractions representing 0.1-0.3% of the total sphingoid bases

Plasma concentrations of total deoxy-sphingolipids (dSL) were significantly higher for the non-diabetic metabolic syndrome group (0.11 µmol/l ±0.04) compared to controls (0.06

µmol/l ±0.02) but did not differ between the metabolic syndrome and diabetic groups (Table 1). This elevation remained significant after the Bonferroni correction for multiple testing. In contrast, C_{16}SO levels were found to be significantly lower (p= 0.008) in diabetic patients (6.37 µmol/l ± 2.82) in comparison the non-diabetic metabolic syndrome (8.38 µmol/l ± 2.25) patients. However, this decrease missed the significance limit after Bonferroni correction in which a significance level of 0.05 corresponds to a p value of 0.002. Other sphingoid bases were not significantly different between the three groups.

A correlation matrix of all variables (clinical data, clinical chemistry measurements along with sphingoid base backbone measurements) showed a significant correlation of the serine-based sphingolipids (C_{16}, C_{17} or C_{18} SA or SO and C_{18} SA-diene) with LDL and total cholesterol but less with HDL cholesterol. In contrast, the alanine based dSLs showed a strong correlation to the metabolic syndrome related variables like waist circumference (wcf) and triglycerides (suppl. Figure 2). Therefore, we were interested to see whether the increased dSL levels are associated with hyperglycemia per se or rather with insulin resistance. To address this issue we analyzed plasma, liver and muscle tissue of streptozotocin (STZ) treated rats (Table 2). Although beta cell failure and not insulin resistance is at the base of the STZ rat model we also found significantly elevated dSL levels in plasma and liver of the STZ rats (Table 2, Fig. 1). No dSL's were detected in muscle tissue. This suggests that plasma dSLs are primarily elevated in association with hyperglycemia and probably of hepatic origin.

An orthogonal partial least square-discriminant analysis (OPLS-DA) was used to estimate the importance of the individual variables as discriminating biomarkers. Both models (control vs. non-diabetic metabolic syndrome and non-diabetic metabolic syndrome vs. diabetes) showed good fit and predictive power. Therefore, they are valid for discrimination. (suppl. Table 1).

In score plots (Fig. 2a) clustering into separate groups is still preserved after reducing the whole dataset into a single predictive component (shown on the x axis) and an orthogonal one (shown on the y axis). Loading plots (Fig. 2b) show the weights of each of the original variables to the model and hence its contribution to the disease state. In the control vs. the metabolic syndrome loading plot, triglycerides, doxSA ,doxSO, diastolic and systolic blood pressure contributed to the metabolic syndrome state model (95% confidence interval >0) whereas elevated HDL cholesterol contributed to the control state model (95%confidence interval <0). This confirms the biological and clinical validity of the model since elevated

triglycerides, low HDL cholesterol as well as hypertension are key features of the metabolic syndrome. DoxSA and doxSO were identified as the second and fourth most important contributors to the metabolic syndrome model just next to triglycerides and HDL cholesterol respectively, but above glucose, waist circumference and systolic blood pressure (Fig. 2b and c). In the metabolic syndrome vs. diabetes model, we found elevated fasting glucose and glycated haemoglobin (HbA1c) concentrations together with low C_{16}SO and creatinine levels to be the contributors for the diabetes state model (Fig. 2b).

Variable importance for the projection (VIP) plots (Fig. 2c) show the contribution of each variable to the variation in both the X space and the Y space (hence its correlation with other variables and the control or disease state). A coefficient value >1 signifies that the variable is *important*. For the control vs. metabolic syndrome model, the VIP plot showed for triglycerides, doxSA, HDL cholesterol, and doxSO the highest VIP coefficients. A slight *importance* (VIP coefficient slightly >1) was noticed for diastolic pressure and fasting glucose. For the metabolic syndrome vs. type 2 diabetes mellitus model a significant *importance* of HbA1c, glucose and C_{16}SO was seen. VIP coefficients were also >1 for body mass index, HDL cholesterol and creatinine.

In summary, the OPLS-DA analysis revealed that triglycerides, doxSA, doxSO and HDL cholesterol are the best explanatory variables for the non-diabetic metabolic syndrome while differences in HbA1c, glucose and C_{16}SO were mostly related to the diabetes.

The diagnostic potential of these markers was analyzed in a Receiver Operating Characteristic (ROC) curve analysis (Fig. 3). For the diagnosis of the metabolic syndrome, doxSA and doxSO had an area under the curve (AUC) of 0.875 and 0.842 respectively ($p<0.001$). Moreover, C_{16}SO showed an AUC of 0.282 (corresponding to 0.718; $p<0.01$).

Discussion:

In this study, we compared the plasma sphingoid base composition of healthy individuals with non-diabetic metabolic syndrome and diabetic patients. We found that deoxy-sphingolipids (dSL) are significantly elevated in the plasma of patients with either non-diabetic metabolic syndrome or type 2 diabetes mellitus compared to controls but not different between non-diabetic metabolic syndrome and diabetic patients. In contrast, C_{16}SO levels were lower in diabetic patients but did not differ between controls and

metabolic syndrome patients. Other sphingoid base metabolites were not different between the groups (Table 1).

This indicates that the metabolic changes in the metabolic syndrome and type 2 diabetes mellitus are specifically associated with alterations in some but not all sphingoid base species. Elevated dSL levels were also confirmed in plasma and liver tissue of a type 1 diabetes model (STZ rats) indicating that the observed increase in dSLs is independent of the type of diabetes.

Sphingolipid metabolism can be considered as a metabolic cross point which interconnects fatty acid (acyl-CoA) and amino acid (serine and alanine) metabolism. Serine and alanine formation is thereby linked to glycolysis which forms their precursors 3-phosphoglycerate and pyruvate respectively.

The metabolic syndrome and type 2 diabetes mellitus are associated with the clustering of several risk factors. To delineate the relative contribution of each variable we used a supervised learning approach (OPLS-DA) [24, 25]. This analysis showed that dSL levels have, in addition to triglycerides and HDL cholesterol, the highest explanatory power for the metabolic syndrome. In this context it has to be considered that the metabolic syndrome and type 2 diabetes mellitus are clinically not two completely separated entities. Most diabetic patients also fulfill the criteria for the metabolic syndrome which, by itself, is associated with insulin resistance. In fact, most diabetic patients have presented for many years with the metabolic syndrome before hyperglycemia manifests. The observation that dSL levels are not different between non-diabetic metabolic syndrome patients and diabetic patients suggests that the dSLs are already formed in the pre-diabetic situation when insulin insensitivity is still compensated by increased insulin production and hyperinsulinemia.

In contrast, C_{16}SO together with glucose and HbA1c were the strongest contributors for the diabetes model. C_{16}SO is generated by the use of myristoyl-CoA instead of palmitoyl CoA - a reaction which is primarily catalyzed by the SPTLC3 subunit of SPT [13].

It should be noted at this point that we also found significant levels of C_{17}SO in the analyzed plasma samples. C_{17}SO is considered to be an “unnatural” sphingoid base and therefore sometimes used as an internal normalization standard in lipidomics. However, the identity of C_{17}SO in human plasma was validated by comparison to a commercial, synthetic C_{17}SO standard and also reported earlier by Quehenberger et al. [26]. This indicates that the use of

C_{17}SO as an internal standard in particular for plasma samples has to be taken with precaution.

Currently we do not fully understand why plasma dSL levels are increased in the metabolic syndrome and diabetes. Deoxysphingolipids are almost exclusively present in VLDL and LDL but not in HDL indicating that the dSLs in plasma are primarily of hepatic origin [27]. This view is supported by the observation that dSL levels were elevated in liver and plasma of STZ rats but not present in skeletal muscle. A possible explanation for the increased dSL formation might be an increase hepatic availability of alanine. Recent reports show that lifestyle modifications in the metabolic syndrome are associated with significant changes in the plasma amino acid profiles [28]. In obese individuals increases of both, skeletal muscle output of alanine and hepatic uptake of alanine were seen while serine levels were not changed [18]. Furthermore hepatic glucose uptake is primarily mediated by GLUT2 and hence insulin independent. Hyperglycemic conditions are therefore associated with elevated hepatic glucose levels and an increased glycolytic flux which increases the formation of pyruvate and its anaerobic conversion into either lactate or alanine. Elevated glucose levels could hence increase hepatic alanine levels and thereby dSL generation. This model implies that dSL levels are elevated independent of the type of diabetes which is supported by the observation that dSL levels were also found to be elevated in plasma and liver of a type 1 diabetes animal model (STZ rats). In both conditions even short-term and transient fluctuations in plasma glucose levels might be integrated in an increased dSLs formation which by themselves might have a slow turnover since they are not degraded by the canonical sphingolipid catabolism.

Another interesting aspect is whether increased plasma dSL levels as well as decreased plasma C_{16}SO levels contribute to the pathogenesis of diabetes and its complications. Genome-wide association studies showed a strong association of genetic SPTLC3 variants with alterations in lipid metabolism [29] and increased risk for myocardial infarction [30]. Therefore lower C_{16}SO levels might be directly or indirectly related to the increased risk of diabetic patients for developing cardiovascular complications.

Previously, we showed that the increased formation of dSLs is the pathological cause for the inherited neuropathy HSAN1 [15, 31]. Clinically, HSAN1 closely resembles the diabetic peripheral neuropathy (DPN) which occurs in about 60% of diabetic patients. Both conditions show a late onset, slow progression and length dependent axonopathy. All

peripheral nerves are affected, including pain fibers, motor neurons and autonomic nerves. The degeneration of small sensory fibers results in the loss of pain sensation, which in turn leads to painless injuries. Furthermore, HSAN1 and DPN are both associated with skin ulcers, which is not a common feature in other peripheral neuropathies. Considering the neurotoxic properties of dSLs, it might therefore be conceivable that the increased dSLs formation is not only interesting from the aspect as biomarkers but also as potential pathogenic agents in the diabetic peripheral neuropathy (DPN). Interstingly, it has been shown recently that serum triglycerides correlate with the progression of the diabetic neuropathy [32]. In parallel we observed a strong correlation of the dSLs and triglyceride levels (suppl. Figure 2).

Taken together our results suggest that dSLs are relevant biomarkers for both the metabolic syndrome and type 2 diabetes mellitus whereas C_{16}SO bases seem to specifically lowered in diabetes, possibly reflecting the risk for developing cardiovascular complications. Therefore, a combination of these markers might help to improve the risk prediction and therapy monitoring of diabetic patients. This could be especially relevant in the transition phase of a pre-diabetic to diabetic state. A limitation of this study is the rather small group size. However, for a pilot biomarker study it is valid to use small numbers as long as type I and type II errors are considered carefully. We showed p value in the order of 1e-5 after the most conservative correction for doxSA and doxSO which goes very well with rejecting the null hypothesis. However, larger and ideally prospective follow-up studies are needed to further validate the potential of these metabolites and to explore their diagnostic and prognostic value. Finally, a more detailed knowledge of the physiological and pathophysiological properties of these metabolites is important to better understand the interplay between sphingolipids, carbohydrate metabolism, insulin resistance and diabetes.

Author contributions: AO, MFR and DE did the lipid extraction and analysis, CHS, PR and HD were involved in study design, sample collection and patient characterisation, CPS , GLP , RP did the STZ rat experiments. AO did the statistical analysis, AvE contributed to study design, data interpretation and TH was involved in study design, data interpretation and wrote the manuscript. All authors approved the final version to be published

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	Control (n=25) Mean±SD	Metabolic Syndrome (n=25) Mean±SD	Diabetes (n=25) Mean±SD	KruskalWallis p-value	Mann-Whitney U p-value C vs MetS	Mann-Whitney U p-value MetS vs D
AGE	61.19±4.28	62.13±5.02	61.56±4.64	0.771	0.50	0.567
Female (%)	40	40	40	1.000		
Smoking (%)	64	56	60	0.846		
Wcf (cm)	98.24±12.10	105.84±8.23	110.80±11.97	3.70E-04	0.004	0.051
BMI	28.19±4.49	30.13±3.01	32.21±4.83	0.006	0.05	0.09
Fast. Gluc. (mmol/l)	5.26±0.56	5.64±0.57	9.59±4.16	1.94E-09	0.03	4.29E-07
HbA1c (%)	5.60±0.33	5.76±0.34	7.79±2.04	4.71E-10	0.17	9.52E-08
Chol(mmol/l)	5.26±1.24	4.99±1.30	4.98±1.44	0.777	0.49	0.861
LDL(mmol/l)	3.35±0.96	3.45±1.00	3.16±1.22	0.449	0.52	0.269
HDL(mmol/l)	1.80±0.69	1.15±0.21	1.31±0.32	1.60E-05	2.37E-06	0.132
TG(mmol/l)	1.10±0.37	2.51±0.83	2.21±1.47	1.96E-07	1.38E-08	0.067
sysBP (mmHg)	126.80±12.47	133.84±15.23	135.52±15.92	0.081	0.12	0.44
diasBP (mmHg)	78.80±8.50	83.92±9.41	82.24±8.99	0.161	0.06	0.565
AST	28.56±8.60	30.84±24.10	25.84±8.43	0.388	0.38	0.662
ALT	29.92±15.71	34.76±24.54	31.68±13.25	0.650	0.35	0.907
Crea	0.84±0.18	0.92±0.21	0.81±0.23	0.144	0.20	0.06
GFR	100.65±11.53	95.26±15.30	100.77±15.68	0.316	0.22	0.184
C16SO (µmol/l)	8.31±4.49	8.38±2.25	6.37±2.82	0.041	0.55	0.008
C16SA (µmol/l)	0.23±0.13	0.23±0.10	0.26±0.14	0.749	0.84	0.491
C17SO (µmol/l)	4.66±2.42	4.02±1.10	3.71±1.48	0.387	0.64	0.29
C17SA (µmol/l)	0.15±0.07	0.14±0.05	0.14±0.05	0.960	0.82	0.961
SO (µmol/l)	88.83±27.23	81.66±21.28	81.54±23.89	0.622	0.34	0.793
SA (µmol/l)	2.17±0.9	2.20±0.77	2.57±1.14	0.473	0.82	0.299
C20SO (µmol/l)	0.20±0.08	0.19±0.06	0.19±0.05	0.727	0.46	0.839
doxSO (µmol/l)	0.15±0.09	0.23±0.09	0.24±0.13	2.14E-04	3E-05	0.839
doxSA (µmol/l)	0.06±0.02	0.11±0.04	0.12±0.05	3.92E-06	5E-06	0.808
C18SA-dienine (µmol/l)	20.79±8.17	18.27±5.74	16.42±6.80	0.148	0.36	0.273

Table 1. Baseline characteristics and results. Values are expressed as means or percentage for scale or categorical variables respectively. P-values were calculated with Kruskal-Wallis test for all three group - and with Mann-Whitney-U for two group comparisons. After Bonferroni correction a p value of 0.002 corresponded to a significance level of 0.05. For the categorical variables asymptotic two sided p-values for the Chi-square are shown. (C for controls, MetS for the metabolic syndrome group and D for the diabetic patients group)

	Liver (pmol/100 µg prot)		Muscle (pmol/100 µg prot)		Plasma (µmol/l)	
	CTRL	STZ	CTRL	STZ	CTRL	STZ
Glucose (mmol/l)	-	-	-	-	8.8 ± 0.4	53.8 ± 3.3**
C16SO	3.4 ± 0.8	3.1 ± 0.7	1.5 ± 0.23	1.4 ± 0.24	0.06 ± 0.02	0.09± 0.07
C17SO	15.7 ± 3.3	13 ± 2.5	4.2 ± 0.44	5.0 ± 1.2	0.3 ± 0.1	0.4 ± 0.1
C18SA-dienine	64.1 ± 10.6	78.1 ± 26.1	11.2 ± 2.3	21.5 ± 10.3*	2.6 ± 0.4	5.0 ± 0.9*
SO	1130 ± 189	1127 ± 252	180 ± 25.4	262 ± 106*	24.3 ± 7.6	29.1 ± 4.8
SA	40.3 ± 9.4	66.4 ± 29*	9.1 ± 1.8	8.1 ± 2.6	1.0 ± 0.5	1.6 ± 0.6*
C20SO	10.8 ± 4.4	11.4 ± 2	6.5 ± 0.6	6.7 ± 1.6	0.1 ± 0.08	0.15 ± 0.9
doxSA	0.2 ± 0.08	0.4 ± 0.12**	n.d	n.d	0.01 ± 0.003	0.12 ± 0.9**
doxSO	n.d	n.d	n.d	n.d	n.d	n.d

Table 2. Results of STZ rat tissue and plasma analysis.

Values are shown as mean±SD, n.d. = non detected, P-values were calculated with Mann-Whitney-U test (* p-value <0.05, ** p-value < 0.001)

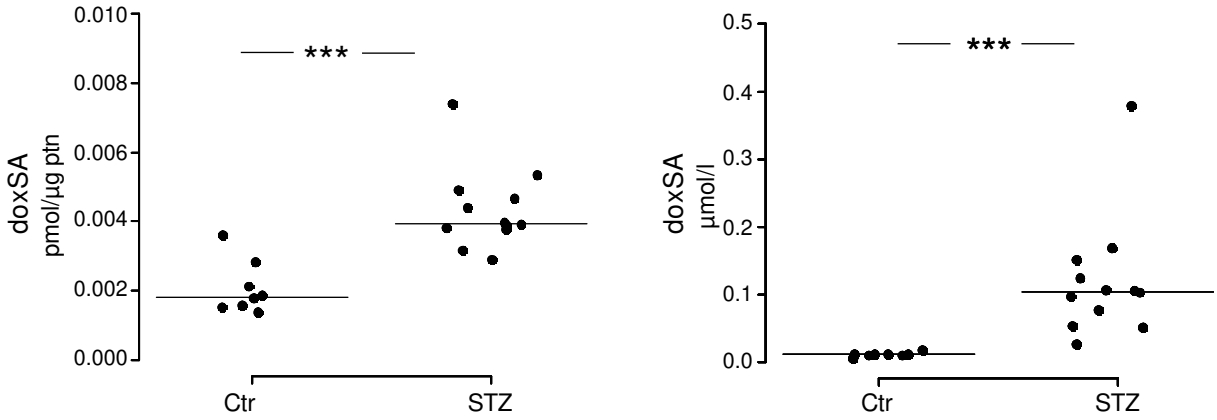

Figure 1 Deoxysphinganine (doxSA) levels are significantly ($p<0.001$) elevated in liver tissue (left) and plasma (right) of STZ rats (STZ) compared to controls (Ctr).

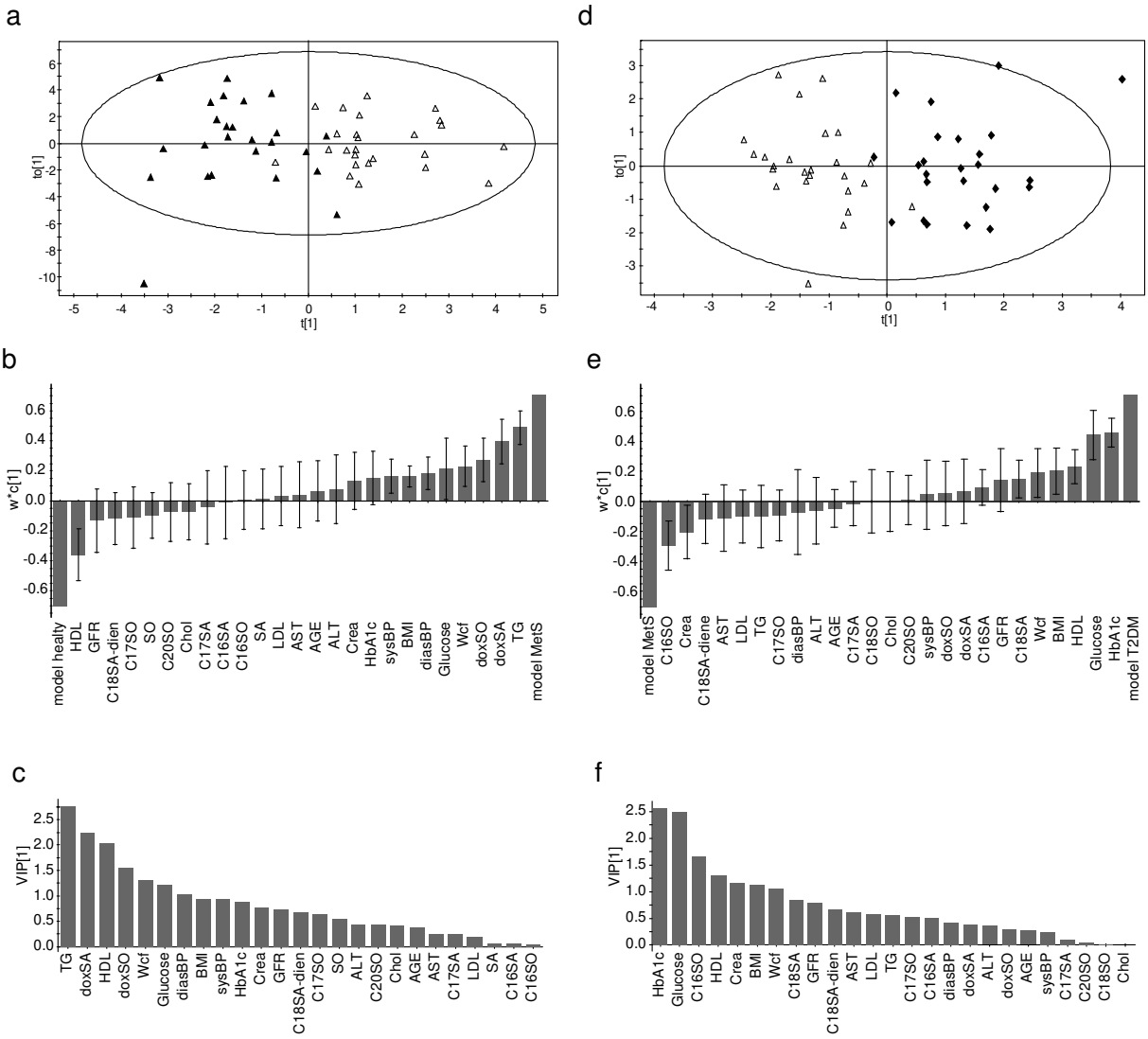

Figure 2 a) Score plots of OPLS-DA models Control (Contr) vs. non-diabetic metabolic syndrome (MetS) (left) and non-diabetic metabolic syndrome (MetS) vs. type 2 diabetes (T2DM) (right). Individual observations are shown as black triangles for controls, open triangles for the metabolic syndrome and black rhombus for type 2 diabetes. On the x axis, the scores for each subject in the predictive principal component (t(1)) is shown, while the y axis shows the scores for each subject in the orthogonal component. Thus, variations on the x axis reflect the between-group separation while variations on the y axis show the within-group variations (noise). The tolerance ellipse corresponds to 95% of the Hotelling`s T^2 multivariate distribution. b) Loading column plots of OPLS-DA models control vs. metabolic syndrome (left) and metabolic syndrome vs. diabetes (right). The weights represent the contribution of each variable to the model component scores. Variables with larger weights contribute more to the model. Error bars represent 95% confidence intervals for calculated weights. c) Variable importance to the projection (VIP) plot for OPLS-DA models control vs.

metabolic syndrome (left) and metabolic syndrome vs. diabetes (right). The VIP coefficients plot shows the summation of all the weights for each X variable to predict Y and hence denoting the *importance* of each X variable.

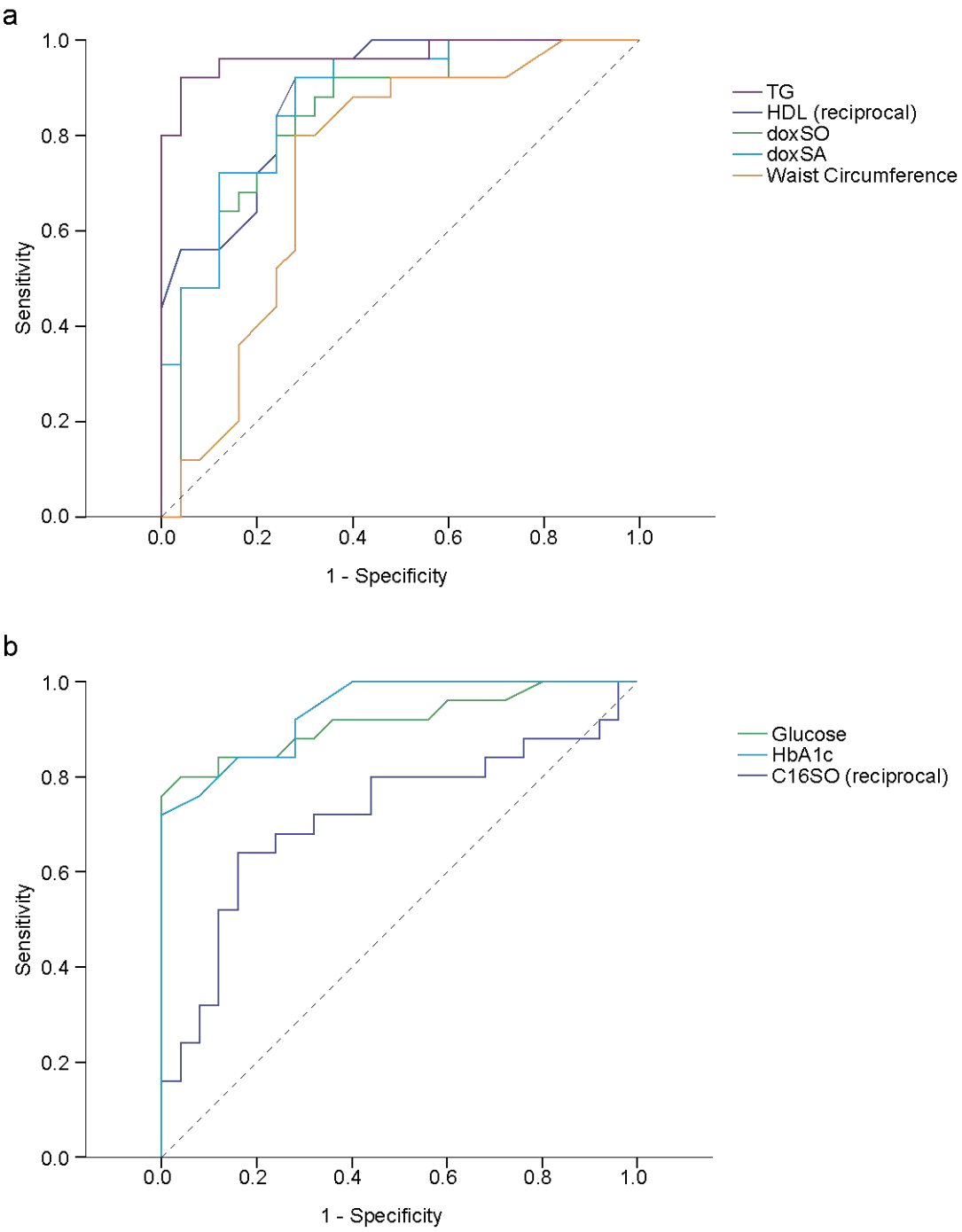

Figure 3 Receiver Operating Characteristics curves (ROC) a) ROC curves for control vs. metabolic syndrome showing triglycerides and HDL cholesterol as predictors of the metabolic syndrome with AUC's of 0.968 ($p<0.001$) and 0.111 (corresponding to 0.889), respectively. The dSLs show comparable AUCs with 0.875 for doxSA and 0.842 for doxSO. b) ROC curves for potential biomarkers in metabolic syndrome vs. diabetes. HbA1c and glucose are showing an AUC of 0.939 and 0.917 for type 2 diabetes whereas $C_{16}SO$ shows a significantly lower AUC of 0.282 (corresponding to 0.718) denoting its decrement in association with diabetes.